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14. ABSTRACT

The purpose of this project is to identify and confirm negative regulator proteins that suppress ErbB2 expression in normal breast epithelial cells, but permit ErbB2 overexpression when dysregulated by tumor cells. The strategy is to use a very sensitive quantitative PCR method to assess transcript levels of candidate modulator genes in normal and tumor tissue from patient samples and mouse models. In the first year of this three year project, PCR primers were designed and initially characterized in terms of their abilities to specifically amplify target genes. In addition, screens of candidate ErbB2 negative regulator genes were initiated using human and mouse tissue samples. The preliminary data from FvB transgenic mice that overexpress ErbB2 in the mammary gland indicate that several genes are consistently upregulated or downregulated during the normal-to-tumor transition. These observations indicate that our overall strategy is likely to ultimately identify dysregulated ErbB modulators in human tumors, the focus of year two of the project.

15. SUBJECT TERMS
breast cancer, ErbB2 overexpression, kRT-PCR

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Table of Contents

INTRODUCTION

Overexpression of growth factor receptors of the ErbB family, including HER1/EGFR, HER2/ErbB2/neu and HER3/ErbB3, contributes to the growth and progression of breast tumors. Much effort has gone into understanding the mechanisms by which overexpressed ErbB receptors contribute to cellular proliferative and invasive properties, which has led to the development of ErbB-directed therapies such as trastuzumab (Herceptin) and lapatinib (Tykerb). However, very little is known about the mechanisms underlying ErbB protein overexpression in tumors. Certainly the HER2/ErbB2 gene is amplified in a subset of breast tumors. However, our observations with transgenic mouse models of ErbB2-induced tumors indicate that augmentation of ErbB gene expression in the mammary gland is not sufficient to give rise to ErbB protein overexpression. We have observed that while ErbB2 and ErbB3 proteins are very highly expressed in mammary tumors of transgenic mice, protein levels in normal or unaffected tissue are very low, despite similar transcript levels. Moreover, two proteins known to suppress ErbB protein levels are present in uninvolved mammary tissue but suppressed in tumors from transgenic mice. Hence, it appears that tumor cells must inactivate very potent endogenous post-transcriptional mechanisms, such as protein degradation pathways, that keep ErbB receptor protein levels in check. While the impact of such ErbB negative regulatory mechanisms in cancer has been largely overlooked, the development of methods to augment or replace these pathways in tumors could offer novel therapeutic strategies to patients whose tumors are resistant to ErbB-directed therapies. Our understanding of ErbB negative regulatory pathways remains in its infancy. Dozens of genes have been identified that could possibly play roles in the trafficking of ErbB receptors for degradation, and dozens more exert a negative effect on receptors through unknown mechanisms. However, roles for these genes in cancer remain to be delineated.

Our goal in these studies is to identify and characterize ErbB negative regulator genes that are suppressed in mammary tumors in transgenic mouse models and in breast tumors from patients. Since traditional microarray approaches have not proven fruitful, we are employing a powerful high throughput kinetically-monitored reverse transcription-polymerase chain reaction (kRT-PCR) approach to evaluate the expression of over 60 ErbB negative regulatory genes in normal and tumor tissues.

The overall project falls into three Specific Aims:
Specific Aim 1 – Develop kRT-PCR primers and protocols to assess human and mouse ErbB negative regulator genes. Months 1-6.

Specific Aim 2 – Analyze the loss of expression of ErbB negative regulatory genes in a mouse model of ErbB2-induced breast cancer as well as in human primary breast tumor samples. Months 7-24.

Specific Aim 3 – Determine whether identified dysregulated genes impact ErbB2-dependent breast tumor cell proliferation or invasion. Months 25-36.

In the current year our efforts have largely focused on Aims 1 and 2.

BODY

A. Primer synthesis and controls. We have used PrimerSelect, a program developed by our collaborator Dr. Michael Holland (Graham and Holland, 2005), to design two sets of polymerase chain reaction (PCR) primer pairs each to 70 candidate genes that may be involved in regulating protein levels or activity of the ErbB2 receptor tyrosine kinase. We have analyzed these for efficiency and specificity using three tests. First, we analyzed the abilities of each of the primer pairs to form primer dimers, which markedly interfere with the efficiency of the PCR reaction, by gel electrophoresis. Primer pairs that showed a high degree of primer dimer formation were not carried forward into further analysis. Second, we assessed the abilities of each of the primer pairs to amplify products from reverse-transcribed RNA isolated from the bacterium *H. pylori* and the yeast *S. cerevisiae*. These microorganisms should not have transcripts of sufficient homology to mammalian genes such that specific PCR products should be amplified. Any primer pairs that exhibited specific bands or non-specific smears as judged by gel electrophoresis after PCR were not carried forward into subsequent analysis. Finally, we analyzed the abilities of each primer pair to specifically amplify a PCR product of the correct size from a mixture of reverse-transcribed RNAs isolated from heart, brain, skeletal muscle, and normal mammary gland tissues. Analysis of publicly-available transcript expression databases reveals that the 70 genes of interest are present in at least one of these tissues. Primer pairs that yielded a unique PCR product of the correct size were carried forward in our analysis; primer pairs that yielded multiple bands or smears as judged by gel electrophoresis were not carried forward for further analysis. After these controls were carried out, primer pairs representing 43 mouse genes were found to be of sufficient quality to move forward in our studies; primer pairs representing 46 human genes were deemed to be of sufficient quality.

B. ErbB receptor regulator expression in mammary tissue obtained from MMTV-ErbB2 transgenic mice. Using the optimized PCR primers for the 43 mouse genes outlined in Part A, we carried out kinetically-monitored RT-PCR (kRT-PCR) reactions on RNA isolated from normal or tumor mammary tissue from transgenic mice. It has been known for over twenty years that amplification of the ErbB2 gene in tumors is common to roughly a quarter of breast cancer patients (Slamon et al., 1987). When its gene is amplified the ErbB2 protein is overexpressed, and countless studies suggest that hyper-signaling by the overexpressed ErbB2 receptor leads to the malignant properties of ErbB2 overexpressing cells.

In an effort to develop a mouse model of ErbB2-amplified breast cancer, Muller and colleagues created a transgenic mouse that specifically overexpressed the ErbB2 cDNA in the epithelium of the mammary gland using the murine mammary tumor virus (MMTV) promoter (Guy et al., 1992). These animals develop spontaneous, focal tumors with a latency of ~25 weeks that metastasize to the lung with high frequency (Siegel et al., 1999. The MMTV-ErbB2 mouse line has been the subject of intense study in efforts to understand the molecular processes underlying ErbB2-induced breast cancer. Curiously, our lab has observed that when normal (non-tumor) mammary tissue from these transgenic animals is compared with wild type mice, similar levels of ErbB2 protein is observed, even though ErbB2 message is over an order of magnitude higher in the transgenic animals (Miller et al., 2008). Moreover, it is only when mammary tissue goes to tumor that ErbB2 protein is overexpressed. These observations strongly suggest that normal mammary epithelial cells harbor potent mechanisms that suppress receptor overexpression. Such mechanisms probably evolved to suppress receptor overexpression to prevent aberrant cellular growth during development and tissue maintenance. More unexpectedly, the dramatic increase in ErbB2 protein expression during the transition of normal tissue to tumor occurs in the absence of changes in transcript levels when transcript levels are normalized to epithelial cell content with an epithelial marker such as cytokeratin. These observations suggest that post-transcriptional mechanisms such as the control of protein synthesis and degradation critically controls ErbB2 receptor protein levels in cells, and these mechanisms are disrupted in tumors. These observations provide the basis of our hypothesis that the dysregulation of genes involved in receptor trafficking and degradation markedly contributes to ErbB2 protein overexpression in tumors.

We have analyzed the expression of the 43 mouse ErbB regulatory genes for which we developed reliable primer pairs. In these experiments eight MMTV-ErbB2 mice were sacrificed, mammary tumor and adjacent normal tissue was excised from each animal, and RNA was prepared from tissues. kRT-PCR reactions on 1 ng, 2 ng or 5 ng RNA was carried out according to our previously established protocols (Holland, 2002; Watson et al., 2004), relative transcript abundance was quantified, and the ratio of transcript for normal tissue relative to tumor tissue was calculated determined for each gene. The results are summarized in **Table 1**. Some transcripts were undetectable in both normal and tumor tissue using our primers and protocols. In other cases aberrant PCR amplification curves made interpretation of the data difficult. However, we were able to obtain reproducible data on the majority of mouse normal/tumor pairs, and observed that expression of the majority of genes did not significantly change during the normal-to-tumor transition. There were several notable exceptions, genes whose expression either increased (green) or decreased (red) upon progression to tumor, and these will be the focus of analysis in subsequent years of the project (see below). In addition, we will assess the expression of these genes in normal and tumor tissue derived from other transgenic mouse models. Our prediction is that the dysregulation of ErbB receptor modulator genes may be specific to the MMTV-ErbB2 model; there is no need for dysregulation of these genes in cases where the driving oncogene is independent of or downstream of receptor signaling.

C. ErbB receptor regulator expression in breast tissue obtained from breast cancer patients. We have begun to analyze the expression of ErbB modulator genes in samples obtained from breast cancer patients treated at the UC Davis Cancer Center. For patient samples, in some cases we have matched normal and tumor tissue, but for the majority of cases we obtained only tumor tissue. This limitation alters our strategy to some degree relative to the mouse studies in that we will need a large number of patient tumor samples to compare to as many normal samples as we are able to obtain. Moreover, in this series of studies it will be beneficial to separately analyze tumors that were scored as ErbB2-positive in the pathology report and those that scored negative. Thus far we have been developing kRT-PCR protocols with human tissue using 5 ng, 10 ng and 20 ng RNA, and we have obtained reproducible data on 12 tumor and three normal samples using the 46 primer pairs that were carried forward from step A. Year 2 of the project will focus on building a deep database of normal and tumor ErbB modulator gene expression patterns, and the ultimate comparison of these data sets.

D. Future directions. In the coming fiscal year we will begin to more closely examine the candidate ErbB regulatory genes that were identified in the MMTV-ErbB2 mouse model. Examination of **Table 1** reveals that in addition to ErbB receptors, Nedd4, Spry1, LRIG2 and SOCS5 are consistently elevated during the normal-to-tumor transition, while Cav1, DCN1 and SOCS3 are suppressed. Future studies will first seek to confirm these trends by immunoblotting mouse and human tissue samples. Those gene products that reproducibly change upon tumor development will be analyzed biochemically to assess their effects on ErbB receptor stability and their impact on tumor cell proliferation, survival and invasive properties.

In addition, we will strengthen our analysis of human tissues, in an effort to identify reproducibly changing transcripts as identified in the mouse model. While trends in dysregulated gene expression were immediately apparent from as few as eight mice, samples from patients will undoubtedly require more extensive analysis because of the genetic heterogeneity in the human population. Our kRT-PCR analysis my be complemented by the analysis of publicly-available databases of broad gene expression patterns in breast cancer patient samples. While these data are far less quantitative, they still have the potential to identify genes that are involved in receptor trafficking and stability whose dysregulation may promote ErbB receptor overexpression and aberrant activation.

Primer pair #	Gene:	Mouse ID: NXN54	NXN9	NXN141	NXN64	NXN46	NXN66	NXN136	NXN145
531	EPS15	2.30	2.30	1.74	0.76	2.30	1.23	4.29	0.54
532	CAV1	0.12	0.14	0.03	0.03	1.32	0.04	0.08	0.01
534	LRIG1	0.93	2.83	1.62	0.71	0.57	1.74	3.25	1.07
537	OTUB1	1.52	0.76	1.23	1.23	0.71	0.93	2.83	0.87
539	RGS4	1.52	ud	ud	ud	ud	ud	ud	ud
541	SOCS4	2.30	4.00	2.14	1.15	1.00	3.73	4.92	1.62
543	ERRFI1	1.15	0.25	4.29	3.73	0.57	1.52	8.00	1.32
544	SPRY4	5.28	11.31	3.48	2.64	1.07	7.46	3.73	1.62
546	MET	1.00	4.00	1.23	1.87	0.87	2.64	4.59	0.38
548	N4BP3	TIP	TIP	TIP	TIP	TIP	TIP	TIP	TIP
550	BIRC6	4.29	4.00	3.03	1.32	1.52	1.41	4.59	1.07
552	NEDD4	14.93	14.93	9.19	5.66	6.96	<0.25	13.93	1.41
555	ERBB2	32.00	29.86	2.83	4.29	2.83	13.93	>20	>20
557	RNF41	TIP	TIP	TIP	TIP	TIP	TIP	TIP	TIP
558	USP8	2.83	5.28	4.29	1.07	0.57	0.71	3.03	1.15
561	DNAJA3	ud	ud	ud	ud	ud	ud	ud	ud
562	LRIG2	ud	ud	1.41	<0.3	ud	ud	ud	>4.0
564	CBLC	ud	ud	3.73	0.01	ud	11.31	8.57	ud
567	SOCS3	ud	3.73	ud	ud	ud	ud	ud	ud
568	SOCS5	ud	>0.5	ud	ud	ud	ud	ud	1.52
571	SPRY3	0.93	2.83	<0.25	0.57	0.87	ud	0.33	0.50
573	CBL	0.31	1.23	0.57	1.00	0.62	2.83	2.64	0.47
574	SPRY2	0.71	0.62	3.73	4.92	1.32	1.15	7.46	4.29
577	LRIG3	1.41	<0.25	1.62	2.14	1.32	1.07	9.19	1.07
579	EGFR	<0.5	0.93	0.54	2.14	6.06	4.92	>2.0	3.25
580	ERBB3	<0.5	1.32	7.46	9.85	2.64	4.00	24.25	3.73
582	SPRY1	TIP	TIP	TIP	TIP	TIP	TIP	TIP	TIP
584	SOCS1	TIP	TIP	TIP	TIP	TIP	TIP	TIP	TIP
589	SNX1	3.03	0.54	3.48	2.83	2.83	3.03	2.30	1.87
591	DCN	0.03	0.06	<0.01	0.02	0.10	<0.01	<0.005	<.02
593	CBLB	0.93	1.23	0.19	0.27	0.41	2.83	0.76	0.08
596	ERBB4	ud	ud	17.15	27.86	6.96	5.28	>2.0	0.41
557	RNF41	0.62	0.31	3.03	0.62	1.41	2.30	1.41	1.15
582	SPRY1	3.25	8.57	1.62	5.28	3.03	3.73	5.66	2.00
584	SOCS1	0.62	0.31	3.03	0.62	1.41	2.30	1.41	1.15
571	SPRY3	0.76	2.83	0.10	2.64	1.41	2.46	0.76	ud
596	ERBB4	3.25	0.02	0.03	4.92	0.47	4.92	4.29	ud
562	LRIG2	4.59	4.92	4.29	4.92	1.87	14.93	ud	3.73
564	CBLC	1.52	1.62	4.29	4.29	0.76	3.03	17.15	2.83
539	RGS4	ud	ud	ud	ud	ud	ud	ud	ud
567	SOCS3	0.22	1.23	0.22	0.23	0.41	0.13	0.16	0.29
568	SOCS5	0.47	8.57	4.59	7.46	3.03	17.15	29.86	2.30
561	DNAJA3	ud	ud	0.50	0.47	ud	ud	1.23	ud

Table 1. Changes in gene expression during the normal-to-tumor transition in ErbB2 transgenic mice. After birth, FvB strain mice bearing the MMTV-ErbB2 transgene were aged for 20-30 weeks to allow ErbB2-induced tumors to develop. Animals were sacrificed, tumor (T) and adjacent normal (N) tissue was harvested, and RNA was isolated from tissues. kRT-PCR analysis of 1, 2 or 5 ng RNA was carried out, and the ratio of the transcript level from tumor tissue to that of normal tissue from eight animals is plotted is presented for each gene. Green represents genes whose transcripts reproducibly increase during the normal-to-tumor transition, while red represents genes that are reproducibly suppressed. ud indicates that transcript undetectable; TIP indicates that technical problems prohibited data interpretaion.

KEY RESEARCH ACCOMPLISHMENTS

- PrimerSelect program was used to design pairs of PCR primers to human and mouse versions of ~70 gene transcripts, including ErbB receptors, genes involved in negative ErbB regulation and genes involved in membrane protein trafficking and degradation.

- Primers were synthesized and purified in-house.

- Primer pairs were assessed by gel electrophoresis for spontaneous primer dimer formation.

- Primer pairs corresponding to 34 human and mouse genes were confirmed in their ability to specifically and uniquely amplify target transcripts.

- Matched tumor and uninvolved tissue obtained from FvB strain mice overexpressing ErbB2 were analyzed for expression of 43 ErbB receptor and putative ErbB negative regulator genes.

- Human breast tumor and normal tissue were analyzed for expression of 43 ErbB receptor and putative ErbB negative regulator genes.

REPORTABLE OUTCOMES

None.

CONCLUSION

From our work thus far we conclude that kRT-PCR profiling is capable of identifying reproducible differences in transcript levels of key proteins involved in modulating ErbB receptor signaling activity. From our analysis of MMTV-ErbB2 mice we have identified several genes whose expression is dysrregulated during the normal-to-tumor transition. Future studies will be aimed at confirming that the gene products are indeed dysregulated in tumors, and if so, how this dysregulation affects ErbB receptor levels and the growth and invasive properties of breast tumor cells. In addition we are beginning to develop similar data using human samples. Future studies will be aimed at shoring up these data sets, identifying dysregulated genes, confirming aberrant gene product presence, and assessing their roles in tumor progression.

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APPENDICES

None.